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16. ABSTRACT

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Cells, proteins, and certain physical-chemical properties of brook trout *(Salvelinus fontinalis)* blood

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Laboratory brook trout were used to evaluate, refine, or develop biochemical procedures for the analysis of fish blood. Analytical values were obtained for the following blood properties: total and differential leucocytes and erythrocytes; erythrocyte and plasma proteins (by electrophoresis); plasma refractive index; erythrocyte sedimentation rate; erythrocyte osmotic fragility; blood surface tension and density; haemoglobin; and packed cell volume. These blood factors are discussed with reference to fish health and disease and to changes caused by deleterious quantities of water pollutants.

I. INTRODUCTION

Knowledge of the composition of fish blood and of the function of blood components is fundamental to a comprehensive understanding of the normal and pathological biology and biochemistry of fish. Such information is particularly important when interpreting the changes induced by water pollutants or other abnormal environmental factors. To establish reliable diagnostic blood and tissue tests for identifying systemic and metabolic dysfunctions of fish, more fundamental information is needed on normal fish blood and tissue, fish metabolism, and the variability of metabolic components.

This report presents a study and review of some analytical procedures applicable to the study of certain fish blood factors that may be relevant to bio-physiological responses of fish to toxic water pollution and adverse environmental conditions. Some of the procedures have not been thoroughly investigated in fish.

The following blood factors were analyzed in adult brook trout (*Salvelinus fontinalis*) maintained in the laboratory: total and differential leucocytes (thrombocytes, lymphocytes, and neutrophils) and erythrocytes (mature and immature); erythrocyte proteins and plasma proteins (by electrophoresis); plasma refractive index; erythrocyte sedimentation rate; erythrocyte osmotic fragility; blood surface tension; blood density; haemoglobin; and packed cell volume.

II. PROCEDURES AND DISCUSSION

MAINTENANCE SYSTEM AND BLOOD SAMPLING

Brook trout (*Salvelinus fontinalis*) weighing from 0·7–1·2 kg were obtained from a hatchery, in Minnesota, and were maintained in untreated Lake Superior water at 10° C in experimental tanks for two months before haematological testing. The

standard physical system and laboratory environmental régime, e.g., water characteristics, temperature, diet, have been described by McKim & Benoit (1971, 1974). Four male and four female fish were studied, each in separate tanks, to provide the maximum number of samples that could be processed promptly in one day by available personnel. From each fish blood specimen all samples were analyzed at least in duplicate. The fish were anaesthetized with ethyl-m-aminobenzoate methanesulfonate (100 mg/l), and the blood was withdrawn with heparinized instruments from the dorsal aorta-post cardinal vein.

LEUCOCYTE ANALYSIS

The analysis and differentiation of leucocytes have wide application in human clinical studies, providing indices of metabolic abnormalities, disease conditions, states of adaptation, immunochemical changes, and physiological defense processes. In fish, however, the significance of this group of cells and their biological function are not clearly understood. Workers disagree on the identification of different leucocytes, largely because of the differences in number and morphology of these cells between species and even within a species. The failure of the extrapolation to fish of the system of identification established for higher animals and humans is due to the inherent differences between fish and mammalian cells.

The leucocytes of a wide variety of marine fish were analyzed by Saunders (1966*a*, *b*, 1968*a*, *b*). Analyses of fresh water fish blood cells have been carried out on the silver salmon (*Oncorhynchus kisutch*) (Katz, 1949); yellow perch (*Perca flavescens*) (Yokoyama, 1947); rainbow trout (*Salmo gairdneri*) (Klontz, 1959); mountain whitefish (*Prosopium williamsoni*) (McKnight, 1966); and the pike (*Esox lucius* L.) (Mulcahy, 1970).

Fish leucocytes respond to various stressors, including infections and chemical irritants (Reichenbach–Klinke, 1966). Weinreb (1958) reported that turpentine (at concentrations causing 30% mortality) caused an increase in heterophils and a decrease in lymphocytes and thrombocytes.

For the quantitative analysis of leucocytes and erythrocytes, the procedure that enabled us to best distinguish between red and white cells (since are all nucleated) was that described by Klontz (1959) but derived from Yokoyama (1947). Some modifications were made in the method. The 'Yokoyama' diluting fluid (part 1) consisted of NaCl (800 mg), KCl (40 mg), 40% formalin (10 ml), dextrose (250 mg), $NaHCO_3$ (50 mg), and distilled water (40 ml). We mixed this solution with an equal part of filtered aqueous Wright's stain (0·2 g 100 ml^{-1}). Blood was diluted 1 : 200 with the modified diluting fluid, and replicate total erythrocyte and total leucocytes counts were made.

For differential staining the slides were fixed in methanol for 5 min and then stained. The specimens were treated for 2 min with commercial Leishman's stain, water washed, and treated for 5 min with Giemsa's stain. The Giemsa's stain solution consisted of Giemsa's powder (1 g), glycerin (66 ml), and methanol (66 ml), which was then mixed (3·5 ml) with phosphate buffer (50 ml, pH 6·4, 0·1 M). The slides were finally rinsed with phosphate buffer and then with water and were then dried in air. Typical cells are shown in Plate I.

Brook trout lymphocytes are round with a deep blue to purple eccentric nucleus that fills most of the cell (Wright's stain). The small band of cytoplasm is medium blue with lighter areas, granular, and usually shows pseudopod formations. The

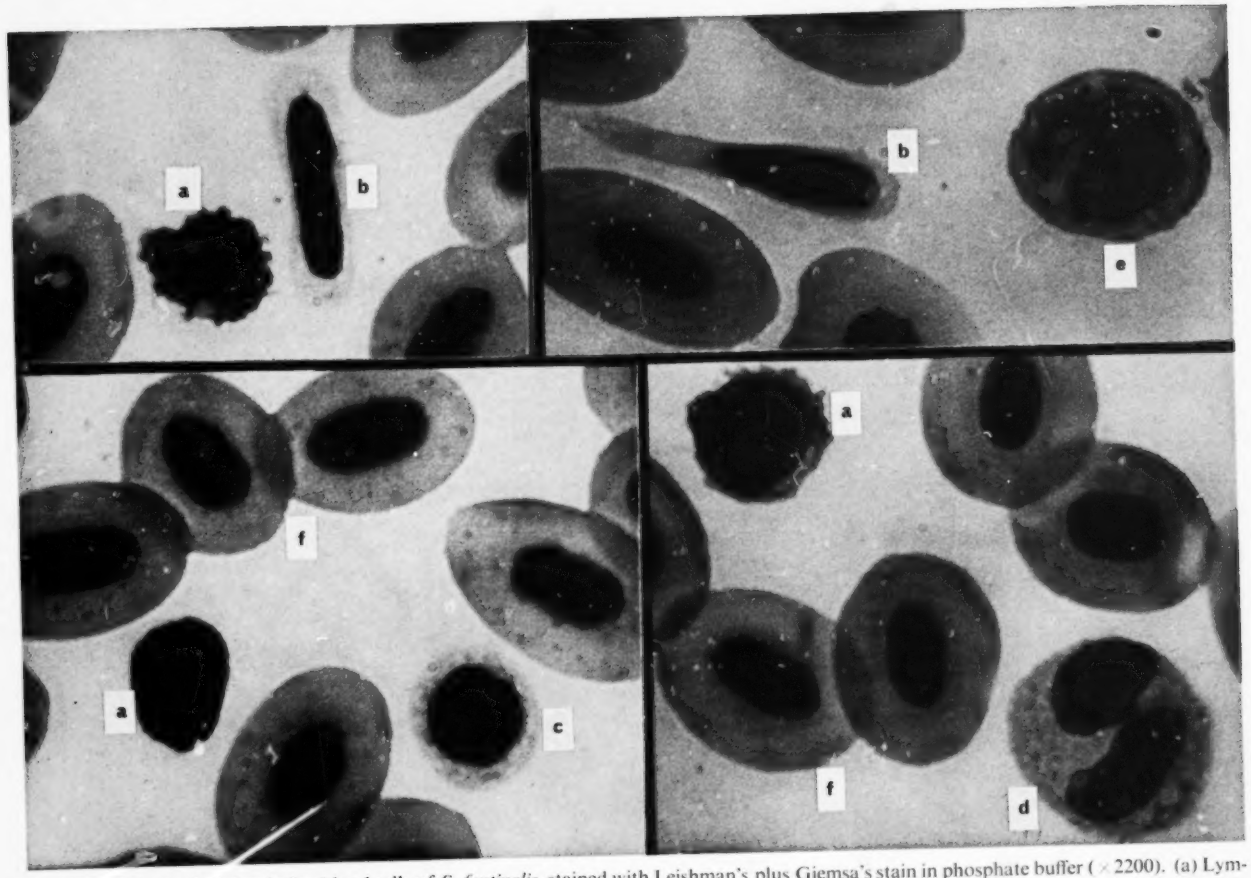

PLATE 1. The principal circulating blood cells of *S. fontinalis*, stained with Leishman's plus Giemsa's stain in phosphate buffer (×2200). (a) Lymphocytes (some with pseudopods); (b) elongated thrombocyte; (c) oval thrombocyte; (d) neutrophil (polymorphonuclear leucocyte); (e) unidentified cell (believed to be an early leucocyte—1% of total leucocytes); (f) mature erythrocytes.

whole cells average 7·8 by 9·1 μm. Thrombocytes are usually elongated with a similarly shaped nucleus which is dense and deep blue. The cytoplasm is pale blue and sometimes has a 'tail' at one or both ends. The entire cell averages 5·0 by 15·1 μm; the nucleus measures 3·5 by 10·7 μm. A less common round cell (about 9 μm) with about the same proportion of nuclear and cytoplasmic material and about the same colouration as the elongated thrombocytes is believed to be an immature form. The polymorphonuclear neutrophil cells are relatively large and round, averaging 11·0 by 12·9 μm with a multi-lobed nucleus (two–six lobes). Early forms have a band-shaped nucleus that sometimes appears indented. The nucleus is deep blue with a pale blue-grey to pink cytoplasm.

ERYTHROCYTES

Erthrocytic responses in fish exposed to toxic chemicals have been noted by McKim, Christensen & Hunt (1970) and Christensen *et al.* (1972). Usually the number of circulating red cells increases, presumably in response to chemical effects upon haematopoietic tissue. In the current study the polychromatic (presumably immature) erythrocytes were measured with the mature cells. The early stage of these cells has largely been ignored although they might be particularly useful indices of toxic stress. If haematopoietic tissue is malfunctioning, the newly generated cells may be malformed or be prematurely released into the general circulation, as in myelophthisic anaemia.

The mature erythrocytes were counted in the usual way (Todd, Sanford & Wells, 1953).

Mature red blood cells are oval with a large oval nucleus. The cytoplasm is a light pink and the nucleus a deep intense blue. The immature or polychromatic cells have a blue cast to the cytoplasm and a deep blue-purple nucleus; these cells have more loosely packed chromatin. Both types of cells average 14·5 μm long by 10·2 μm wide; the nucleus measures 4·1 by 7·2 μm. The immature red blood cells were counted in the following manner; two differential slides from each fish sample were examined for polychromatic red blood cells by counting a total of 1000 cells to obtain a percentage of immature cells present. The results of the blood-cell analyses are shown in Table I. Photographs of the principal red and white cells are shown in Plate I.

BLOOD-PROTEIN ELECTROPHORESIS

Fish blood has been subjected to extensive protein analyses by electrophoresis, and a number of reports have been published (Briere & Mull, 1964; Summerfelt, 1966). However, we found no information concerning brook trout. There is no clear parallel between the brook trout electrophoretic blood pattern and the patterns of several other fish species that we have examined. Although high precision can be obtained in electrophoresing a control standard for human blood, which is much different from fish blood, the precision was much less with both fish blood plasma proteins and lysed erythrocyte proteins. We are unable to explain this lack of precision at this time. The protein patterns of fish blood are known to change rapidly in quality and quantity under a variety of stimuli. Among the many factors that have been cited as contributing to this variation, the principal ones are: sex, age, seasonal change (Poston, 1970), disease state (Thomas & McCrimmon, 1964; Mulcahy, 1969), toxicity (Neuhold & Sigler, 1960), and intraspecific hybridization (Tsuyuki & Roberts, 1965). A review of the variations found in fish serum proteins has been prepared by Booke (1964).

TABLE I. Mean and range values for various brook trout blood factors

Factor	Male		Female	
	Mean	Range	Mean	Range
Packed cell volume (%)	57·7	56–61	61	55–65
Haemoglobin (g%)	14·1	12·5–14·8	13·1	11·4–14·0
Surface tension (dynes/cm)	5·299	5·384–5·141	4·984	4·434–5·215
Refractive index	1·3418	1·3405–1·3426	1·3422	1·3416–1·3437
Density (g/ml)	0·1041	0·1038–0·1043	0·1040	0·1033–0·1057
Sedimentation rate (Initial) (% distance in mm/min)	1·423	1·251–1·761	1·866	1·583–2·256
Osmotic fragility (% NaCl causing 50% lysing)	0·367	0·35–0·42	0·344	0·30–0·40
Erythrocytes				
Total (No/mm³)	$1{\cdot}22\times10^{6}$	$0{\cdot}96–1{\cdot}63\times10^{6}$	$1{\cdot}11\times10^{6}$	$87–1{\cdot}34\times10^{6}$
Immature (% of total erythrocytes)	0·5	0–0·6	0·57	0·2–1·0
Leucocytes				
Total (No/mm³)	$2{\cdot}70\times10^{4}$	$1{\cdot}6–3{\cdot}9\times10^{4}$	$2{\cdot}20\times10^{4}$	$1{\cdot}60–2{\cdot}85\times10^{4}$
Lymphocytes (% of total leucocytes)	36·2	25–63	53·7	32–50
Thrombocytes (% of total leucocytes)	57·0	32–68	34·1	15–67
Neutrophils (% of total leucocytes)	5·9	3–10	11·6	1–29
Other (% of total leucocytes)	0·4	0–2	0·4	0–2

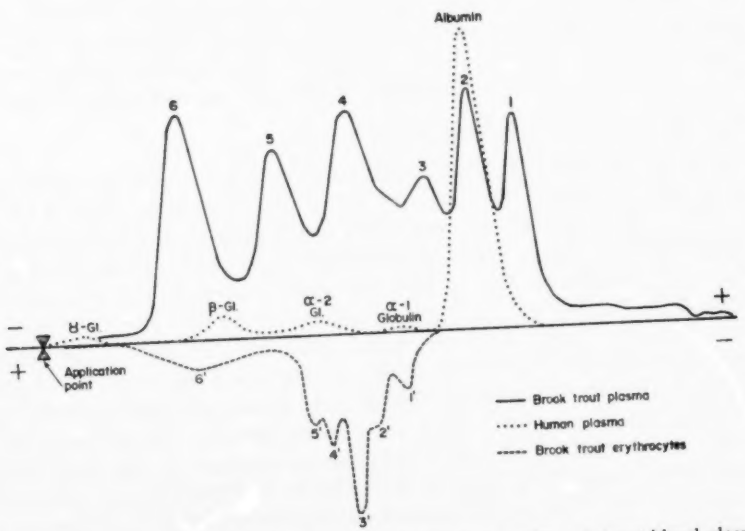

FIG. 1. Typical densitometer tracings from paper electrophoresis of brook trout blood plasma and lysed erythrocyte proteins and human blood plasma proteins.

By isolating or eliminating these causes of variation, we hoped to get a better understanding of the inherent blood-pattern variation among individual brook trout. The animals were therefore separated according to sex, were of the same approximate age, from the same genetic stock, and sampled at as nearly the same time as possible.

The electrophoretic analyses were performed on $2{\cdot}5 \times 10$ cm cellulose acetate strips (Sepraphore III) with a Gelman elecrophoresis tray and power supply. Plasma was run with tris-barbital buffer, pH 8·8, and ionic strength 0·05, and lysed erythrocytes were run with tris-EDTA-borate buffer, pH 8·7, ionic strength 0·05. Plasma samples were applied at the cathode end of the strips and subjected to 300 V for 45 min. Erythrocyte samples were applied at the anode end of the strips and analyzed at 350 V for 35 min. The remaining materials and procedures were followed according to published methods. Quantitation of the cleared strips was accomplished by means of the Gelman Digiscreen-M optical scanner and integrating recorder.

TABLE II. Results of brook trout and human blood plasma electrophoresis

Sample	Fraction 1 albumin	2 α-1-globulin	3 α-2-globulin	4 β-globulin	5 γ-globulin	6
Relative percentage						
Human	64·2	2·6	9·4	12·6	11·1	
Trout	12	15	10	26	17	21
Mobility (mm)*						
Human	150	127	98	69	19	
Trout	171	154	140	117	93	62

* See text for explanation.

For a comparison of the brook trout plasma with a human reference standard, both types of samples were applied to a single strip with a dual applicator and treated with 300 V for 45 min. A typical densitometer tracing is shown in Fig. 1; numerical values are shown in Tables II and III. The mobilities are given in millimeters of distance migrated from the point of application to the center of a given peak as measured on the densitometric tracing. The area under a given peak was calculated in terms of the relative percentage of the total area (protein). Although some brook trout fractions can be seen to nearly coincide with specific fractions in the human samples, a number of reports (Westfall, 1943; Weinreb, 1963) indicate that it would be unwise to assume any strict correlation between human and other mammalian protein fractions on the basis of mobility alone. For this reason the plasma and erythrocyte fractions studied in this experiment were simply assigned numbers starting with the fraction migrating the greatest distance from the point of application. For the plasma proteins six distinct peaks were almost always discernible, whereas the erythrocyte proteins invariably separated into two groups. The first (fastest-moving) group usually showed five distinct peaks, and the second (slow-moving) less distinctly resolved group showed from one to three peaks.

Table III summarizes the results of the experimental trout analyses. Eight fish were studied, four male and four female. The *t*-statistic for the difference between two

means was applied to test any sexual differences. For six degrees of freedom and with a two-tailed test, the experimental t value must exceed 2·45 for significance at the 95% level. The mobility of the female plasma proteins is significantly greater than the male mobility for all six fractions (Table III). In spite of the effort to control or eliminate sources of variation, individual fish varied widely in relative percentage protein measurements, whereas the variation in mobility values was not nearly as great. The source of this variation must be considered at this time as inherent in the individual animal's response to its environment.

The lack of a clear distinction between albumin and globulins in this species negates the analysis of differentiated proteins based strictly upon the methodology for the analysis of human proteins (viz. albumin-globulin ratios). Because many water toxicants such as heavy metal cations can be expected to bind strongly to fish blood proteins causing changes in their electrophoretic profile, electrophoretic results could provide indices of toxic stress to aquatic animals.

TABLE III. Results of electrophoresis of brook trout blood plasma and erythrocyte proteins

Fraction	Concentration (% of total)				Mobility (mm)			
	Male		Female		Male		Female	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Plasma								
1	14	11–17	11	10–12	154	148–159	176	170–181
2	18	16–20	16	14–19	135	130–138	153	146–157
3	12	5–19	12	9–16	113	108–122	141	130–146
4	14	6–25	19	10–26	98	90–107	117	108–124
5	18	12–20	20	18–22	77	74–79	91	82–98
6	24	23–25	24	21–28	48	44–50	60	50–67
Erythrocyte								
1'	6	5–7	7	4–11	120	115–127	114	109–118
2'	11	9–13	11	9–12	107	104–113	103	98–108
3'	24	18–32	26	24–28	100	96–105	95	91–101
4'	26	21–31	22	13–30	91	87–98	87	83–93
5'	12	11–14	16	11–18	84	79–91	81	76–87
6'	22	10–32	20	15–28	38	31–47	42	36–46

REFRACTIVE INDEX

The refractive index of the blood plasma of three species of fish has been reported (Altman & Dittmer, 1961): cod (*Gadus morhua*) at 7·5° C r.i.=1·34215; plaice (*Pleuronectes platessa*) at 17·5° C r.i.=1·34165; and tench (*Tinca tinca*) at 17·5° C r.i.=1·34089. The measurement of refractive index, surface tension, and density, gave additional information of the physical-chemical properties of brook trout blood. The plasma was analyzed with an Abbe refractometer at 25° C, and multiple readings were taken for each sample. The results are shown in Table I.

ERYTHROCYTE SEDIMENTATION RATE

The sedimentation rate of erythrocytes of rainbow trout has been reported by Barnhart (1969) and McCarthy, Stevenson & Roberts (1973, 1975). An increase in

rate was observed in brook trout with furunculosis infection (Schumacher, Hamilton & Longtin, 1956). This blood property is measured in human clinical medicine as an aid in the diagnosis of inflammatory lesions, tuberculosis, rheumatic fever, and several other disorders (Frankel & Reitman, 1963*a*).

The blood samples were drawn into 100-µl capillary tubes, and one end was sealed with clay and a critocap. The samples were placed in a centrifuge at 5° C and rotated at 500 rev min^{-1} (35 g). The relative rate of sedimentation was then determined at 5, 15, 30, 60, 120, and 180 min. Final centrifugation at 4500 g for 5 min was then carried out to provide a value for the packed cell volume.

From an examination of the rate curves, the equation of the line was assumed to be $D=L(1-e^{-rt})$, with a slope therefore of $R=rLe^{-rt}(t=0)=rL$, where L=packed cell volume; D=distance (actually the relative distance of the cell-plasma interface from the surface of the plasma); t=time in minutes; R=slope; and r=the initial slope when $t=0$. By plotting ln $(1-D/L)$ against t, the (approximate) initial slope was obtained. These results are given in Table I.

ERYTHROCYTE OSMOTIC FRAGILITY

Increased osmotic fragility in humans is found with chemical poisoning and burns; decreases occur with thalassemia, sickle cell anaemia, erythraemia, and jaundice (Frankel & Reitman, 1963*b*). Normal values for a number of mammalian species are available (Altman & Dittmer, 1961). In *Esox lucius* L. a normal mean value of 0·32% of NaCl (which causes a 50% lysing of red blood cells) was reported (Mulcahy, 1970).

The method used here was based upon a procedure in which the percentage of haemolysis is plotted against the percentage of NaCl. The volumes used were 25 µl blood per 2·50 ml of saline (% v/v NaCl of 0, 0·2, 0·3, 0·4, 0·4, 0·6, and 0·85); the test temperature was 1° C. After mixing, the samples were kept on ice for 1 h and were then centrifuged (2000 *g* for 15 min at 1°) and the absorbance read at 540 nm. Standard curves for haemoglobin were prepared for each concentration of the saline solutions as the molar extinction coefficients varied slightly with different saline solutions. Thus, the cell fragility was expressed by the concentration of haemoglobin dispersed in the aqueous samples, and the haemoglobin value was expressed by the concentration in water. For each sample the percentage of NaCl solution causing the initial and the final degree of haemolysis was recorded. The final fragility values shown in Table I refer to a calculated value of NaCl whereby 50% of the sample was haemolysed. All individual fish blood samples were analyzed in duplicate and average values determined.

DENSITY

The specific gravity of whole blood is known for humans and for a number of other vertebrates (Altman & Dittmer, 1961). Westfall (1943) found that blood specific gravity fell markedly when goldfish (*Carassius auratus*) were subjected to anoxic stress for 1 h. Using the falling drop densitometer, Abegg (1950) measured the effects of inorganic salt solutions on blood and tissue fluid specific gravity of the bluegill (*Lepomis macrochirus* Raf.). The values reflected a change in the physiological condition of the catfish (*Ictalurus punctatus*) (Higginbotham & Meyer, 1950). The blood specific gravity decreased in rockfish (*Sebasticus marmoratus*) that had develop ulcers because of chlorine in the test water (Yamashita, 1967). Haws & Goodnight

(1962) tested many blood factors including the specific gravity in two species of catfish in relation to their habitat. Values for seven fish species were given by Saito (1954).

Specific gravity is usually measured by the falling drop method (Barbour & Hamilton, 1926) or by the cupric sulfate drop method (Phillips *et al.*, 1950). In the current study blood was measured into 100-µl pipets at 25°. The sample weight was obtained, and the densities were calculated. Results are given in Table I.

SURFACE TENSION

The published surface tension value for human whole blood is 55·5–61·2 dyn cm^{-1} (Altman & Dittmer, 1961). The surface tension of fish blood was measured in duplicate by the capillary rise method at room temperature by drawing into 25-µl micropipettes placed vertically in small 1-ml test tubes. Two pipettes were used together to obtain one value; in one the blood was allowed to rise, and in the other the blood was drawn up and allowed to fall. After about 5 min with very gently tapping, the two levels were nearly at the same height, and an average value for height was determined. The radius of the tubes was determined from a microscopic measurement of a cross section of the tubing (r=0·0355 cm). The surface tension (σ) was then calculated: $\sigma = 1/2\ (hdgr)$, where h=height of column (cm); d=density of the blood (g cm^{-3}); g=gravity at 980·67; and r=tube radius (cm). The final value is expressed in dynes per centimetre (Table I).

HAEMOGLOBIN

The haemoglobin value of many species of fish has been determined and is known to be a useful index of health. The haemoglobin changes caused by the cupric ion reported by McKim *et al.* (1970) for the brook trout and by Christensen *et al.* (1972) for the brown bullhead *Ictalurus nebulosus* may have been the result of such an effect. Lead is known to inhibit delta-aminolevulinic acid dehydratase, an enzyme involved in haemoglobin synthesis (Fiandt, pers. comm.). Haemoglobin values for the brook trout are given in Table I.

PACKED CELL VOLUME

Packed cell volume determinations are available from normal and abnormal fish. In particular, the haematopoietic generating system evidently can be disrupted by water toxicants causing abnormal haematocrit and packed cell volume values (Hesser, 1960). Our values, obtained by the standard centrifugation procedure, are shown in Table I.

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